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The Influence of pH and Various Anions on the Distribution of NH_4^+ in Human Blood

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Summary: The pH-dependent distribution of ammonia between blood cells and plasma was investigated with oxygenated blood samples from healthy subjects at 37 °C. Blood pH was varied between 6.95 and 7.65 by equilibration with different CO_2 mixtures. Plasma ammonia concentrations were measured directly with a specific enzymatic method. Ammonia concentrations within blood cells were calculated from a) the concentration changes of ammonia in plasma after addition of 87.7 µmol/l NH_4Cl to whole blood and b) the pH-dependent haematocrit. The pH-dependency of the distribution $ratio_{ammonia}$ = P-ammonia/cell ammonia (substance concentrations in water spaces) is described by the equation distribution $ratio_{ammonia} = 3.095 - 0.342 \times pH_{plasma}$ ($r = 0.928$, $n = 36$) in good agreement with available literature data on the distribution of H^+. A quantitative figure to describe the actual NH_4^+ concentration in oxygenated whole blood at defined values of P-NH_4^+, P-pH and haematocrit is given. Values of distribution $ratio_{ammonia}$ at pH 7.4 (0.57 or 0.75, ammonia concentrations corrected/not corrected for water content) are higher than those assumed so far in the literature. Addition of non-permeating anions (citrate, EDTA) to whole blood results in a shift of NH_4^+ from the intra- to the extracellular compartment. In contrast, chaotropic anions like iodide or thiocyanate lower distribution $ratio_{ammonia}$. To avoid medically important bias in the measurement of plasma ammonia concentration, the changes in pH or in the ionic composition of the blood sample following pretreatment with anticoagulants or preservatives should not exceed certain limits. Citrate is not a suitable anticoagulant.

Introduction

Several mechanisms sustain the in vivo disparity of the ammonia[1]) concentrations in cells, tissues and body fluids of the organism. Concentration gradients between adjacent compartments may result from ammonia producing and/or consuming metabolic reactions, transport phenomena, membrane or diffusional limitations and, finally, from trapping NH_3 as NH_4^+ in the compartment with lower pH (1, 2). For the distribution of ammonia across the blood-brain barrier it has been shown that the normal brain/blood concentration ratio of 1.5 to 3.0 is at least temporarily changed if the energy metabolism of the brain is actually disturbed. Therefore it is not always possible to predict brain ammonia concentrations with certainty from ammonia concentrations determined in blood (1).

Measurements of the ammonia concentration in whole blood or separated plasma are mostly thought to be diagnostically equivalent for the detection and monitoring of hyperammoniaemic syndromes (3). The observed correlation between the ammonia concentrations in whole blood and plasma (4) can be explained by the well-known fact that NH_3 equilibrates within approximately 100 milliseconds across red cell membrane (5). However, the ratio of ammonia concentrations present in plasma and cells in thermodynamic equilibrium

$$\text{distribution ratio}_{ammonia} = c_{plasma}/c_{cells}$$

[1]) The term "ammonia" is used for "total ammonia" = NH_3 + NH_4^+.

and also the relation between the ammonia concentrations in whole blood and plasma cannot be regarded as constant for the following reasons: About 98% of total ammonia exist as NH_4^+ at physiological pH ($pK'_a \sim 9.1$ at 37 °C (6)). Distribution $ratio_{ammonia}$ is therefore nearly completely determined by the pH-dependent *Gibbs-Donnan* effect and accordingly varies with pH as was pointed out decades ago (7). Furthermore, one has to expect that the distribution of NH_4^+ is influenced by certain salts, as is the case with H^+ or Cl^- (8, 9). Recently it has been suggested that the marked changes of distribution $ratio_{ammonia}$ observed after exhaustive muscular exercise may occur secondary to acidosis and an increase of the lactate concentration in plasma (10). Similar shifts of ammonia between erythrocytes and plasma may occur in vitro if blood samples for ammonia analysis are treated with dipotassium EDTA which lowers blood pH (11). Thus the use of this recommended anticoagulant (12) could even become a source of analytical bias.

Little has been published on the distribution of ammonia in human blood. Furthermore, these data were obtained with rather unspecific micro-diffusion methods (4, 13) or a somewhat problematic enzymatic method (cf. l. c. (14)) for the analysis of acid filtrates from whole blood (10). The present study of the ammonia distribution in human blood makes use of a very specific method for ammonia measurement in plasma described before (12). Values of distribution $ratio_{ammonia}$ under various experimental conditions were calculated from the observed concentration changes of ammonia in plasma after adding defined amounts of NH_4Cl to whole blood. In this way the influence of the temperature- (11) and pH-dependent (15) process of ammonia formation in blood was excluded and the distribution experiments could be performed at the physiologically relevant temperature of 37 °C. The results presented may be interesting from both a physiological and an analytical standpoint.

Methods

Subjects

Venous blood samples were obtained from 10 male and 9 female volunteers (age between 26 and 75 years) who were mainly recruited from hospital personnel and gave their informed consent. All appeared to be healthy and exhibited no abnormal results in a routinely performed test profile except for slight elevations of γ-glutamyltransferase (EC 2.3.3.2) or alanine aminotransferase (EC 2.6.1.2) activities in 3 cases. Blood samples were taken between 9 and 12 a.m. in the non-fasting state.

Reagents

Heparin solution, 5000 × 10^3 I.U./l (Liquemin 5000) was purchased from Hoffmann La Roche AG, Grenzach-Wyhlen, Germany. EDTA tripotassium salt cat. no. 3665 was from Fluka Chemie AG, Buchs, Switzerland. Sodium salts of citrate (cat. no. 6447), iodide (cat. no. 6523) and thiocyanate (cat. no. 6627) were obtained from Merck AG, Darmstadt, Germany. Reagents for enzymatic ammonia determination were prepared as described earlier (11). Any ammonia contamination of the solutions was considered by using appropriate blanks.

Analytical procedures

Plasma ammonia concentrations were measured in duplicate with the NADPH-dependent enzymatic method (12) according to an endpoint procedure (16) that was adapted to the bichromatic Abbott VP analyser (11). Accuracy was checked within each run using stable control solution (Preciset Ammonia cat. no. 166 570, Boehringer Mannheim GmbH, Mannheim, Germany). Mean bias during the study was −1.3%. The within-run precision for plasma samples was good (CV = 1.6%). Plasma pH was measured with a Corning 178 pH/Blood Gas Analyzer (Corning Medical and Scientific, Corning Glass Works, Medfield, MA, U.S.A.). Mean bias was 0.003 and −0.009 at pH 7.150 and 7.634, respectively (Certain+, Ciba Corning Diagnostics GmbH, Giessen, Germany). With phosphate buffer, the pH of which is traceable to the NIST scale (Precision Buffer Solution Type S 1510, Radiometer Copenhagen, Copenhagen, Denmark), mean bias was 0.00167 at pH 7.383; between-run imprecision was 0.050%. Haematocrit values were determined by 10 min centrifugation of heparinised capillaries in a "micro"haematocrit centrifuge with 12 000 min^{-1} (A. Hettich, Tuttlingen, Germany). Water content of plasma and whole blood was estimated by weighing 300 µl portions before and after drying at 70 °C for 48−72 h. Cell water content was calculated according to the formula: (blood water −((1-haematocrit) × plasma water)/ haematocrit (cf. l. c. (4)); the mean cell water content determined in 19 healthy subjects was 713 g/l (standard deviation = 19 g/l) in accordance with published data (17). Haemoglobinometry and blood cell counts were performed with a Coulter STKS haematologic apparatus (Coulter Electronics Inc., Hialeah, FL, U.S.A.).

Measurement of the pH-dependent distribution of ammonia in blood

Venous blood (25 ml) anticoagulated with 25 × 10^3 I.U./l heparin (Liquemin 5000), were stored anaerobically at 20−22 °C (primary sample). Portions of 6 ml were subsequently equilibrated for 15 min with gas mixtures of 0.02, 0.05, 0.12 or 0.30 l/l CO_2, 0.20 l/l O_2 and supplementary N_2 ((Linde AG, Unterschleißheim, Germany) in an IL 237 tonometer (Instrumentation Laboratory, Lexington, MA, U.S.A.) at 37 °C. Two 2.80 ml portions of the equilibrated blood sample (a, b) were drawn anaerobically into an EDTA-free monovette (cat. no. 03.258, Sarstedt, Nümbrecht, Germany). Sample (a) was spiked with 87.7 µmol/l ammonia by injection of 50 µl 5 mmol/l NH_4Cl in 0.15 mol/l NaCl via the Luer opening of the monovette through a cannula fixed to a multipette (Eppendorf Gerätebau Netheler-Hinz, Hamburg, Germany). As a control, 50 µl of 0.15 mmol/l NaCl solution was added to sample (b). After careful mixing, the blood samples were incubated for 5 min in a water bath at 37 °C. Blood cells were separated by centrifugation (2100 *g*, 5 min) in a centrifuge (Rotanta P, A. Hettich, Tuttlingen, Germany) that was preheated to 37 ± 2 °C. Aliquots of the plasma supernatant were transferred anaerobically into plastic syringes for pH determination. Ammonia concentrations were determined in subsamples stored at −38 °C (cf. l. c. (11)) for up to 5 hours until measurement. Heparin plasma obtained from the residual primary sample was spiked with 87.7 µmol/l to determine $\Delta plasma\ ammonia_p$ (see below). Mean recovery in plasma was 96% of the theoretically expected concentration.

Calculations

It is assumed that ammonia not recovered in the plasmatic compartment on addition to whole blood is contained within the volume occupied by the blood cells. Accordingly, the intracellular concen-

tration of added ammonia (Δcell ammonia) was calculated from the haematocrit value and the measured changes in plasma ammonia concentration after adding 87.7 μmol/l NH_4Cl to separated plasma (Δplasma $ammonia_p$) or to whole blood (Δplasma $ammonia_b$) with the formula:

$$\Delta\text{cell ammonia} = (\Delta\text{plasma ammonia}_p - ((1 - \text{haematocrit}) \times \Delta\text{plasma ammonia}_b))/\text{haematocrit} \quad \text{(Eq 1)}$$

The "direct" distribution ratio of ammonia in blood (without considering the different water content in plasma and cells) is defined by

$$\frac{\Delta\text{plasma ammonia}_b}{\Delta\text{cell ammonia}} = \text{distribution ratio}_{\text{ammonia(direct)}} \quad \text{(Eq 2)}$$

The distribution ratio of ammonia related to the water spaces of plasma and cells is defined by

$$\frac{(\Delta\text{plasma ammonia}_b/\text{plasma water}}{(\Delta\text{cell ammonia})/\text{cell water}} = \text{distribution ratio}_{\text{ammonia}(H_2O)} \quad \text{(Eq 3)}$$

Influence of added salts on distribution $ratio_{ammonia}$ in oxygenated blood

Heparinised venous blood (25 ml) supplemented with 100 μmol/l NH_4Cl was gently shaken with an excess of atmospheric air at 20–22 °C in a 100 ml plastic syringe until ≥ 98.5% oxygenation was achieved. Portions of 2.7 ml were transferred to EDTA-free 3 ml sample tubes closed by a rubber membrane (cat. no. 47.556, Sarstedt, Nümbrecht, Germany). At the end of a 5 min incubation period in a water bath at 37 °C, 25 μl of anticoagulant solution (0.54 or 1.08 mol/l tripotassium EDTA, 1.08 mol/l trisodium citrate) or 0.15 mol/l NaCl solution as control were injected through the membrane. After immediate mixing, plasma was separated anaerobically at 37 °C for measurement of pH and ammonia concentration (see above). Separate blood samples with the same concentrations of added salts were prepared for the determination of haematocrit and water content. Analogously the influence of chaotropic salts (NaCl, NaI, NaSCN) on ammonia distribution was studied by mixing 1 part of 1 mol/l salt solution with 10 parts of oxygenated blood.

Calculation

The actual value for distribution $ratio_{ammonia(direct)}$ in the absence of added salt was computed from the plasma pH according to the empirical regression formula that had been found in the investigation regarding the dependency of distribution $ratio_{ammonia(direct)}$ on pH (see above):

$$\text{distribution ratio}_{\text{ammonia(direct)}} = 3.491 - 0.371 \times \text{pH}.$$

With cell ammonia = P-ammonia/distribution $ratio_{ammonia(direct)}$ the ammonia concentration in whole blood was then calculated according to the equation:

$$\text{B-ammonia} = \text{P-ammonia} \times (1 - \text{haematocrit}) + \text{P-ammonia} \times \text{haematocrit/distribution ratio}_{\text{ammonia(direct)}}. \quad \text{(Eq 4)}$$

The cell ammonia concentration in the presence of added salt was calculated from the measured plasma ammonia concentration and the actual haematocrit as:

$$\text{cell ammonia} = (\text{B-ammonia} - ((1 - \text{haematocrit}) \times \text{P-ammonia}))/\text{haematocrit}. \quad \text{(Eq 5)}$$

Distribution $ratio_{ammonia(H_2O)}$ was then computed with equation (Eq. 3) considering the different cell water concentrations after addition of salt (cf. l. c. (9)).

Results

pH-dependent distribution of ammonia between plasma and blood cells

The recovery of ammonia in plasma after adding small amounts of NH_4Cl to whole blood increased from 83.2% at a mean pH of 7.645 to 98.5% at pH 6.976 (tab. 1). Parallel to these changes in recovery, the distribution $ratio_{ammonia(H_2O)}$ calculated according to equations (Eq. 1) and (Eq. 3) increased from 0.49 to 0.71. The individual values obtained in 9 experiments are shown in figure 1. To describe the pH-dependency of the distribution ratio, linear regression analysis of the data shown in figure 1 was performed yielding the equation

$$\text{distribution ratio}_{\text{ammonia}(H_2O)} = 3.09 - 0.34 \times \text{pH} \quad (r = -0.91, n = 36) \quad \text{(Eq 6)}$$

If ammonia concentrations in plasma or cells (without correction for water space) are considered, the corresponding equation is:

Tab. 1 pH, recoveries of added NH_4Cl in plasma and calculated distribution ratios of ammonia in the equilibration experiments. Mean values (standard error of mean) are given (n = 9). For further details see "Methods".

CO_2 (volume fraction, l/l)	pH	Haematocrit (l/l)	Recovery (%)	Distribution $ratio_{ammonia(direct)}$ [a]	Distribution $ratio_{ammonia(H_2O)}$ [b]
0.02	7.645 (0.004)	0.447 (0.0076)	83.2 (1.48)	0.65 (0.010)	0.49 (0.007)
0.05	7.443 (0.003)	0.455 (0.077)	88.4 (1.20)	0.73 (0.010)	0.55 (0.008)
0.12	7.226 (0.003)	0.464 (0.0078)	93.2 (1.68)	0.81 (0.012)	0.62 (0.011)
0.30	6.976 (0.004)	0.475 (0.0079)	98.5 (1.56)	0.90 (0.016)	0.71 (0.018)

[a] P-ammonia concentration/cell ammonia concentration
[b] ammonia concentration in plasma water/ammonia concentration in cell water

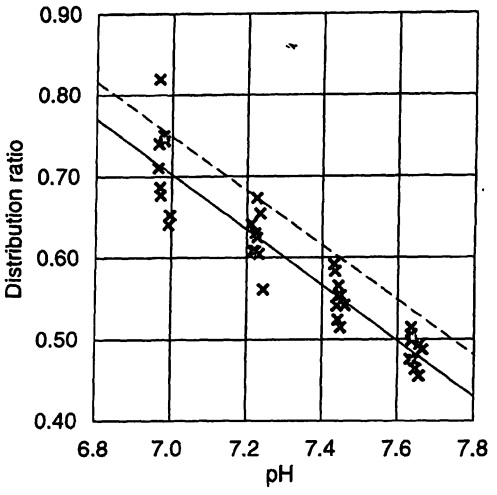

Fig. 1 Distribution of added ammonia (87.7 µmol/l) between the water spaces of plasma and blood cells at different plasma pH values. Regression line for distribution $ratio_{ammonia(H_2O)}$ (−). For comparison, the regression line for distribution $ratio_{H+}$ (---) according to literature data (8) is shown.

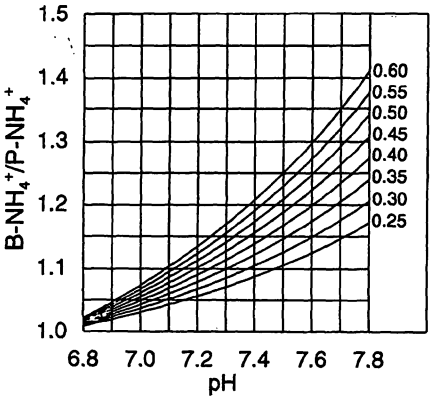

Fig. 2 Relation between the NH_4^+ concentrations in plasma and whole blood: dependence on plasma pH and haematocrit. Numbers on the right end of the curves designate haematocrit values.

$$\text{distribution ratio}_{ammonia(direct)} = 3.49 - 0.037 \times pH \quad (r = -0.94,\ n = 36) \qquad \text{(Eq 7)}$$

Based on this expression, the ammonia content of oxygenated whole blood at 37 °C can be predicted from the plasma ammonia concentration at a given pH and haematocrit according to equation (Eq. 4) (fig. 2). The small and variable fraction of NH_3 gas that can be calculated from the pK'_a (0.005 to 0.05 between pH 6.8 and 7.8) is not considered in the figure.

Influence of various anions on ammonia distribution

As it has been reported that the distribution of H^+ can be influenced by salts (8, 9), we investigated the effect of several substances on the distribution of ammonia. Addition of salts with non-permeating anions (citrate, EDTA) led to increases of distribution $ratio_{ammonia(H_2O)}$ (fig. 3). with 10 mmol/l trisodium citrate, 5 or 10 mmol/l tripotassium EDTA, mean values of distribution $ratio_{ammonia(H_2O)}$ increased by 19.2, 6.7 and 17.0%, respectively, as compared to the controls (distribution $ratio_{ammonia(H_2O)} = 0.55$, pH 7.46, n = 10). This was accompanied by an increase of the plasma ammonia concentrations by 8.5, 2.8 and 7.6%, respectively. The mean pH changes caused by the anticoagulants did not exceed 0.09 pH units. Haematocrit values remained unchanged.

With permeating "chaotropic" monovalent ions (iodide, thiocyanate) applied at much higher concentration, the distribution ratio of ammonia could be manipulated (for intended application see "Discussion") in the opposite direction (fig. 3). Compared to controls (pH 7.41), mean distribution $ratio_{ammonia(H_2O)}$ was decreased by addition of 91 mmol/l sodium iodide or sodium thiocyanate by 10.9 and 14.8%, respectively (n = 5). The lower distribution ratios in the presence of chaotropic anions resulted from an increase of intracellular ammonia concentrations by 14.4% (iodide) or 16.3% (thiocyanate), while haematocrit values decreased, due to an extensive water shift, by 28.3 or 27.1%, respectively. Plasma ammonia concentrations remained virtually unchanged under the conditions applied.

Discussion

For theoretical reasons it has been postulated that the distribution ratios of H^+ and NH_4^+ in blood are identical

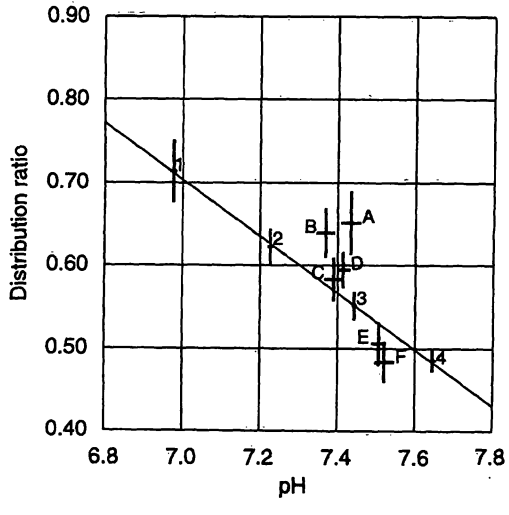

Fig. 3 Influence of pH and added salts on distribution $ratio_{ammonia(H_2O)}$. Mean values and ± 2 SEM ranges are shown.
1–4: blood without salt additions, pH varied by equilibration with CO_2 mixtures;
A–F: oxygenated blood with endogenous pCO_2;
A: 10 mmol/l trisodium citrate;
B: 10 mmol/l tripotassium EDTA;
C: 5 mmol/l tripotassium EDTA;
D: 91 mmol/l NaCl;
E: 91 mmol/l NaI;
F: 91 mmol/l NaSCN.
Number of experiments 9 (1–4), 10 (A–C), or 5 (D–F).

and that pH changes will influence them in the same manner (7). At high concentrations of added ammonia, experimental evidence supporting this theory could be obtained in an early study with bovine blood (7) and later on in experiments with human and avian blood (18). Our own results on the distribution ratios in oxygenated human blood at 37 °C are in satisfying agreement with well-documented data in the literature (8) on the hydrogen ion distribution under similar conditions (oxygenated human blood, 38 °C). As can be seen from figure 1, the slopes of the regression lines found for NH_4^+ (this study) and for H^+ (literature data taken from l. c. (8)) are very similar.

According to equation (Eq. 6) distribution $ratio_{ammonia(H_2O)}$ equals 0.57 at pH 7.4. From the data of *Büttner & Büttner* (19) who measured the intra- and extracellular hydrogen ion concentration in blood by potentiometry or with the 5,5-dimethyl-2,4-oxazolidinedione method, similar values of distribution $ratio_{H+}$ (mean pH 7.40; 37 °C) are calculated: 0.60 (potentiometry) and 0.56 (5,5-dimethyl-2,4-oxazolidinedione method). *Harris & Dudley* (10) determined the ammonia concentrations in the water spaces of plasma and blood cells in six resting volunteers using an enzymatic method. Some overestimation of the cellular ammonia concentration due to the instability of the ammonia concentrations in acid filtrates from whole blood (cf. l. c. (14)) may have caused the somewhat lower distribution ratio (0.48) that can be calculated from their data (10).

For distribution $ratio_{ammonia(direct)}$ equation (Eq. 7) yields 0.75 at pH 7.4. Reported values[2]) of distribution $ratio_{ammonia(direct)}$ (physiological pH, subjects with normal ammonia concentrations) are considerably lower: 0.36 (20), 0.31 (4) or 0.50 (13); it has been stated recently that the ammonia concentration of red blood cells is approximately three times that of plasma (21). The low plasma/cell ratios were obtained with microdiffusion methods that liberate NH_3 on treatment with alkali. At the high pH applied during microdiffusion the rate of ammonia formation from labile sample compounds is higher with whole blood than with plasma (cf. l. c. (22)). Accordingly, ammonia in whole blood is overestimated with these methods in a concentration-independent manner. At higher ammonia concentration, after infusion (6) or oral ingestion (4) of NH_4Cl solutions, the ratios obtained with microdiffusion methods approximate the value found in the present study. From the data of *Buttery* et al. (23), who determined ammonia concentrations in plasma and erythrocytes with an ion exchange method, distribution $ratio_{ammonia(direct)}$ is calculated as 0.70.

The whole-blood/plasma ratio of ammonia (see fig. 2) depends on distribution $ratio_{ammonia(direct)}$ at given pH (equation (Eq. 7)) and the haematocrit value; with haematocrit = 0.45 the whole-blood/plasma ratio is calculated according to equation (Eq. 4) as 1.16 at pH 7.4. This value is markedly lower than the ratio reported in a review (21).

A very high value of distribution $ratio_{ammonia(H_2O)}$ (0.83) has been observed with blood samples taken from volunteers during recovery after supramaximal exercise (10). According to the present in vitro study (equation (Eq. 6)) this exceptional ratio would correspond to an (unplausible) blood pH of 6.6 while the estimation of pH from reported blood lactate concentrations (cf. l. c. (24)) yields approximately 7.0. It has been suggested that a transient disproportionate increase of lactate in plasma may lead to a reduction of the concentration gradients of NH_4^+ and H^+ (10).

After establishing the relation between plasma pH and distribution $ratio_{ammonia}$, the influence of different salts on ammonia distribution could be studied with a simplified experimental procedure as the ammonia concentration in whole blood could be calculated from pH and haematocrit (equation (Eq 4)). Citrate and EDTA do not permeate the red cell membrane and cause an increase of distribution $ratio_{ammonia}$ (fig. 3). The observations with EDTA appear to be especially important as it has been recommended as an anticoagulant for measurement of the plasma ammonia concentration (12). However, the calculated mean increase of the plasma ammonia concentration with 5 mmol/l trisodium EDTA was small (< 3%). From the biological variation of the P-ammonia concentration in a reference population a maximum allowable inaccuracy of 10% has been calculated for this quantity (11) with reference to proprosed quality standards (25). Thus the effect of trisodium EDTA on the distribution of ammonia at usual low concentration (4.5 mmol/l) appears to be tolerable although it is generally desirable to avoid any known source of inaccuracy in analytical procedures. However, with certain reagents for the enzymatic determination of P-ammonia concentration the use of EDTA as anticoagulant is essential (12, 26). 10 mmol/l citrate is by no means recommendable if ammonia determination is intended. 25×10^3 I. U./l heparin used for anticoagulation in this study is not expected to have a measurable influence on ammonia distribution.

In a previous paper it was shown that the problematic stability of the plasma ammonia concentration in blood samples can be improved by decreasing pH (15). However, practical use of this principle (e. g. addition of an acid buffering salt) is limited as it follows from the observed influence of pH on ammonia distribution (see

[2]) Literature data cited were adapted to the definition of the distribution ratio used in the present paper.

fig. 1) that a decrease of the plasma pH value by 0.1 units will cause an increase of the P-ammonia concentration by 2.5% in a blood sample with a haematocrit value of 0.45. "Chaotropic" ions like iodide or thiocyanate are able to decrease the distribution ratio of hydrogen ions in blood (9). The same is true for distribution $ratio_{ammonia}$ (fig. 3). However, addition of chaotropic anions in the order of 10^{-1} mol/l provoked a water shift from the intra- to the extracellular space with concomitant shrinking of the blood cells (see "Results"); a substantial reduction of the ammonia concentration in plasma was not achieved. Therefore it was not possible to counterbalance the effects of acidification by addition of chaotropic anions in a useful manner.

In conclusion, the present work gives further experimental proof that the distribution of blood NH_4^+ between plasma and cells follows the theoretical pH-dependent *Gibbs-Donnan* equilibrium. The dependency of the plasma ammonia concentration from pH and salt additions has to be considered during the preanalytical phase. In order to measure true in vivo P-ammonia concentrations the pH of the blood sample has to be kept unchanged until blood cells are separated. Blood samples should be processed in closed tubes to avoid loss of CO_2. Ionic additives used for the preparation or preservation of blood samples should be carefully checked if they cause a shift of NH_4^+ between plasma and blood cells.

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